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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

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<input type="checkbox"/> Additional inventors are being named on the ___ separately numbered sheets attached hereto.					
TITLE OF THE INVENTION (280 characters max)					
THYMOSIN α 1 ACTIVATES DENDRITIC CELLS FOR ANTIFUNGAL TH1 RESISTANCE THROUGH TOLL-LIKE RECEPTOR SIGNALING					
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

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USE ONLY FOR FILING PROVISIONAL APPLICATION FOR PATENT

**Thymosin α 1 activates dendritic cells for antifungal Th1
resistance through Toll-like receptor signaling**

Abstract

Dendritic cells show a remarkable functional plasticity in the recognition of the fungus *Aspergillus fumigatus* and orchestrate the overall antifungal immune resistance in the lungs. Thymosin α 1, a naturally occurring thymic peptide, induces functional maturation of and interleukin-12 (IL-12) production by both murine and human dendritic cells upon exposure to the fungus. This translates in vivo in the occurrence of protective T-helper cell 1 (Th1)-dependent resistance to aspergillosis in otherwise highly susceptible hematopoietic transplanted mice treated with synthetic thymosin α 1. The activation of p38 mitogen-activated protein kinase/NF- κ B by signaling through distinct Toll-like receptors underlies the ability of the peptide to directly interact with cells of the innate immune system. Our findings reveal the unexpected activity of an old molecule. The ability to act as a natural activator of dendritic cells qualifies thymosin α 1 as a candidate adjuvant promoting the coordinated activation of the innate and adaptive Th immunity to fungi and provides the rationale for its worldwide therapeutic prescriptions.

Introduction

Invasive aspergillosis (IA) is the leading cause of both nosocomial pneumonia and death during the first 100 days after bone marrow transplantation (BMT) (1-3), with an estimated infection rate of 3%-11% in BMT recipients (4). Despite advances in new antifungal agents, IA has an associated mortality rate of >90% if untreated. A recent study on epidemiology of IA in BMT recipients indicated a reduced neutropenia-related infection and an increase "late-onset" infection in the past decade (2, 3, 5), a finding attesting to the importance of the non-neutropenic immunosuppressed state in the pathogenesis of the disease.

Aspergilli are respiratory pathogens, and pulmonary infections are usually acquired through the inhalation of conidia (6). Effector mechanisms of the innate and adaptive immune systems are recognized host defenses against IA (7, 8). Dendritic cells (DCs) orchestrate the overall antifungal immune resistance in the lungs (9, 10). The recent evidence that in healthy individuals and in patients surviving IA a significant antigen-specific proliferation of IFN- γ -producing T cells occurred (11), points to the crucial role of a Th1 reactivity in the control of infection. A dense network of DCs has been described in the respiratory tracts (12-14). In the resting state, respiratory tract DCs are specialized for uptake/processing but not for presentation of antigen, the latter requiring cytokine maturation signals that presumably occur after migration to regional lymph nodes (12, 15). The evidence that pulmonary DCs, through production of IL-10, mediate unresponsiveness to respiratory antigens (16), suggests that the ability of DCs to instruct the appropriate T cell responses to the invading pathogens may be affected by local immuno-regulatory events. In the case of *Aspergillus*, by using distinct pattern recognition receptors, including Toll-like receptors (TLRs), pulmonary DCs were found to be able to finely discriminate between conidia and hyphae of *Aspergillus* in terms of adaptive Th responses elicited after migration to the draining lymph nodes (9, 17). A protective Th1-mediated resistance was induced upon vaccination with *Aspergillus* antigens

and the TLR-9 ligand CpG-ODN as adjuvant (17). These results suggest that the proper manipulation of DC functioning in vivo may translate into beneficial effects in fungal infections.

Thymosin α 1 is a naturally occurring thymic peptide with multiple biological activities on cells of the immune system (18). A synthetic 28-amino acid peptide exhibits efficacy in patients with hepatitis B and C, some immunodeficiencies and malignancies (18, 19). Although thymosin α 1 increased Th1-type responses in patients with hepatitis C (20), its mechanism of action and the specific receptor on T cells involved are still largely unknown. The finding that it activates mitogen-activated protein kinases (MAPK)-transduction pathways (21) and regulates gene expression (22) in macrophages, suggests that the immuno-modulatory activity of thymosin α 1 may occur through an action on resistance mechanisms of innate immunity, as already observed (23).

To verify whether a receptor-mediated signal pathway is activated by thymosin α 1 on DCs, in the present study the effect of thymosin α 1 on functional activity of DCs in response to *A. fumigatus* was investigated. Both murine and human DCs undergo functional maturation and produce IL-12 upon exposure to thymosin α 1 and conidia, the activity being associated with the induction of distinct TLRs expression and p38/NF- κ B activation. This translates in vivo in the occurrence of protective Th1 resistance to IA in otherwise highly susceptible hematopoietic transplanted mice.

Results

Thymosin α 1 activates DCs. DCs are the initiators of the immune response to *Aspergillus* in the respiratory tract (9). Here we show that thymosin α 1 activates lung DCs in response to conidia of the fungus. The simultaneous exposure to conidia and thymosin α 1 induced the activation of DCs, as evidenced by the increased expression of MHC Class II antigens, CD40 and CD86 molecules (Fig. 1A). Thymosin α 1 also increased the production of IL-12 in response to conidia, as indicated by the increase of the frequency of IL-12-producing DCs. IL-10-producing DCs were not similarly increased (Fig. 1B). TLR signaling in monocyte/macrophages (30, 31) and in DCs (18) occurs in response to conidia and hyphae of *Aspergillus* and mediates functional responses to the fungus. To assess whether TLR signaling is also involved in the activity of thymosin α 1, lung DCs were exposed to conidia and/or thymosin α 1 and assessed for TLR expression by RT-PCR. The results show that several TLRs were expressed upon exposure to thymosin α 1, such as TLR2, TLR3, TLR5, TLR8 and TLR9 or to conidia, such as TLR3, TLR4 and TLR8 or to thymosin α 1 and conidia, such as TLR3 and TLR8. However, TLR2 only was uniquely activated by thymosin α 1 in response to conidia (Fig. 2A). Interestingly, the exposure to conidia prevented the expression of TLR5 and TLR9 induced by thymosin (Fig. 2A). No TLRs expression was induced upon incubation of cells with PBS alone (data not shown). As NF- κ B and p38 MAPK activation are early events in triggering TLR-induced gene expression (32, 33), we assessed the nuclear translocation of NF- κ B as well as p38 phosphorylation in DCs exposed to conidia and/or thymosin α 1. Nuclear translocation of NF- κ B was not observed upon exposure to conidia alone. However, the exposure to thymosin α 1, either alone or with conidia, induced NF- κ B activation. Similarly, although a low level of p38 phosphorylation was observed upon treatment with thymosin α 1, a great increase was observed upon the combined exposure with

conidia (Fig. 2B). To determine whether thymosin α 1 also activates human DCs, immature and mature DC1 and DC2 were assessed for phagocytosis of unopsonized *Aspergillus* conidia and cytokine production in vitro in the presence of thymosin α 1. The results show that imDC1 and imDC2 were both able to phagocytose conidia, although to a greater extent for the latter. Similarly, although decreased as compared to the corresponding imDCs, mDC2 showed a higher phagocytic activity than mDC1. The exposure to thymosin α 1 significantly increased the phagocytic activity of imDC1, leaving largely unaffected that of the other DC subsets (Fig. 3). Interestingly, the exposure to thymosin appeared to affect imDC maturation, as indicated by the occurrence of cells with more cytoplasmatic projections, particularly in imDC1. In terms of cytokine production, IL-12 p70 was mainly produced by DC1 and IL-10 mainly produced by DC2, being mDCs more productive than imDCs. Both productions were significantly increased by thymosin α 1 in mDCs. All together, these results point to a novel, previously undefined, immunoregulatory role for thymosin α 1 in the activation and functioning of DCs.

Thymosin α 1 protects BMT-mice from IA. We have previously shown that pulsing lung DCs with fungal antigens in the presence of a TLR-adjuvant resulted in the occurrence of Th1-mediated resistance to IA (17). To assess the effect of in vivo treatment with thymosin α 1 in mice with IA, we resorted to different murine models of infection, including mice immunosuppressed with cyclophosphamide and allogeneic BMT-mice known to be highly susceptible to fungal infections (24, 34). In both models, mice were injected with conidia, treated with thymosin α 1 and assessed for microbiological and immunological parameters of infection. Figure 4 shows that treatment with thymosin α 1 greatly increased resistance to IA, as revealed by the increased survival after the infection and the decreased fungal growth in the lungs. The survival significantly increased in BMT-mice after thymosin treatment (Figure 4A), but full resistance to IA (>60 days survival) was achieved in the majority of

cyclophosphamide-treated mice (Figure 4B). Full protection to IA induced by thymosin α 1 was dose-dependent, being maximal at 400 μ g/kg, a dose at which the therapeutic efficacy of thymosin was superior to that of an optimal dose of amphotericin B (Table 1). Interestingly, thymosin α 1 also synergized with amphotericin B, as indicated by the increase of the therapeutic efficacy of a sub-optimal dose of the antifungal agent (Table 1). The beneficial effect of thymosin α 1 correlated with a decreased inflammatory pathology in the lungs of mice with infection. Lung sections from infected mice showed signs of bronchial wall damage and infiltrates of predominant polymorphonuclear cells scattered throughout the lung parenchyma along with numerous fungal cells (Fig. 5A). These features were not observed in thymosin (Fig. 5B)- treated mice, whose lungs were characterized by few healing infiltrates of polymorpho- and mono-nuclear cells and no evidence of parenchymal destruction and fungal growth.

Thymosin α 1 accelerates functional Th1 cell recovery in mice with IA. In mice with IA, resistance to infection correlates with the activation of IFN- γ -producing Th1 cells (8, 10, 17). To assess whether thymosin α 1 would accelerate Th1 cell recovery in mice with IA, cell recovery was assessed by FACS analysis together with the assessment of antigen-specific lymphoproliferation, pattern of local cytokine production and antifungal activity of effector phagocytes. Cytofluorimetric analysis of lung cells revealed that the numbers of CD4⁺ and CD8⁺ cells, but not of NK cells, were significantly higher in BMT (Fig. 6A) or cyclophosphamide-immunosuppressed (Fig. 6B) mice treated with thymosin α 1 as compared to untreated mice. Interestingly, thymosin α 1 also appeared to increase the number of neutrophils but not that of macrophages. Total and differential counts of blood leukocytes indicated that the absolute number of circulating lymphocytes and neutrophils significantly increased after thymosin treatment (data not shown). Treatment with thymosin α 1 also induced antigen-specific proliferation of CD4⁺ T cells, as revealed by the decreased MFI

value upon CFSE labeling (Fig. 7). To determine the pattern of local cytokine production, the number of IFN- γ - and IL-4-producing CD4⁺ T cells were determined in the lungs of mice with IA upon treatment with thymosin α 1. In both BMT (Fig. 8A)- and cyclofosphamide (Fig. 8B)-treated mice, the frequency of IFN- γ -producing cells greatly increased, while that of cells producing IL-4 decreased, upon treatment with thymosin α 1, as compared to untreated mice. The levels of IL-12 p70 in BAL fluids were also significantly higher in mice treated thymosin α 1 than untreated mice (data not shown). On assessing the level of antifungal activity of effector phagocytes it was found that the conidiocidal activity of both macrophages and neutrophils was higher in thymosin-treated than untreated mice (data not shown). However, as the phagocytosis and killing of conidia by alveolar macrophages and circulating neutrophils from uninfected mice were also significantly potentiated in vitro in the presence of thymosin α 1 (Fig. 9), it follows that thymosin α 1 not only promote DC maturation but will also activate local effector cells for prompt phagocytosis and killing of the fungus.

Results

Discussion

The present study reports on the ability of thymosin α 1 to activate DCs for Th1 priming in aspergillosis. Thymosin stimulates the phagocytosis and the functional maturation of DCs upon exposure to *Aspergillus* conidia and induces the activation of protective Th1-dependent resistance to infection. Complete cure from IA was achieved by treatment with thymosin α 1, an effect comparable to that obtained with amphotericin B. However, treatment with thymosin also potentiated the antifungal activity of effector phagocytes, a finding confirming the benefit of the coordinated activation of the innate and adaptive immune systems in resistance to the fungus (8, 9, 17).

Pulmonary DCs showed a remarkable functional plasticity in the recognition of the fungus (10, 18). Through the use of distinct recognition receptors, DCs finely discriminated between conidia and hyphae and were responsible for the disparate Th responses to them (9). This would be consistent with a certain degree of flexibility of the immune recognition pathways to *Aspergillus* antigens and allergens, such that an allergen could be converted to a potential protective antigen, provided CpG-ODN as adjuvant (17).

It is now clear that Th1 adaptive immune responses require TLR signals (35). Optimal signaling responses to *A. fumigatus* required TLR2 in both mouse and human cells (30), and TLR9 in murine DCs (17). We found here that thymosin α 1 induced the expression of both TLR2 and TLR9 in lung DCs and TLR2 was also induced in the presence of conidia. Therefore, it is likely that an agonist activity on these TLRs may underlie the adjuvant activity of thymosin α 1 in promoting antifungal Th1 responses. This would be consistent with the ability of thymosin α 1 to induce NF- κ B and p38 MAPK activation that mediates transcription of pro-inflammatory genes upon TLR engagement (36). The p38 MAPK signaling pathways are known to regulate a wide range of inflammatory responses, particularly in the lung (37) and in DCs (38). Production of IL-12 was increased in both

human and murine DCs upon exposure to thymosin α 1, although IL-10, and little IL-12, was observed in human DC2. Unlike murine plasmacytoid DCs which produce IL-12 p70 in response to TLR9 stimulation (39), human plasmacytoid DCs are known to produce little IL-12 p70 (27). On a morphological analysis, thymosin α 1 appears to promote the differentiation of imDCs, particularly imDC1, a finding that would be consistent with its ability to induce prostaglandins (40), known to induce the maturation of DCs (41) as well as with its anti-apoptotic activity (42). All these findings suggest that thymosin α 1 could act as a potent adjuvant for both imDC1 and plasmacytoid imDC2. As human lung DCs have a phenotype and functional capacity similar to immature blood DCs (43) and functional heterogeneity is an intrinsic feature of both human (12) and murine (13) lung and airways DCs, it can be proposed that thymosin α 1 may act as a fine modulator of the activation and maturation of different DC subsets at the sites of infection.

It has been proposed that human and mouse plasmacytoid DCs play a central role in antiviral innate immunity (44, 45) and in immune responses after hematopoietic cell transplantation (46). This will explain the beneficial effect of thymosin α 1 in some viral infections (18, 19, 47) as well as on functional hematopoietic cell recovery (48). In this regard, it is remarkable that treatment with thymosin α 1 accelerated lymphoid cell recovery in BMT-mice to an extent similar to that observed upon adoptive transfer of antigen-pulsed DCs (49). This finding, together with the ability to enhance human thymopoiesis (50), points to a possible beneficial effect of thymosin α 1 in promoting immunoreconstitution in hematopoietic transplantation.

TLRs activate the innate immune system not only to assist the adaptive immune system but also for direct antimicrobial effector activity (51). We found that activity of thymosin α 1 not only relied on an effect on DCs but also on effector phagocytes and, in particular, neutrophils that were recruited in the lungs of BMT-mice with IA upon treatment

with thymosin α 1. As massive infiltration of neutrophils is also the hallmark of progressive IA (8), this would indicate that the presence of neutrophils at sites of the infection is neither predictive of the outcome of infection nor of the efficiency of immunoreconstitution. This would be in line with the notion of the existence of different populations of neutrophils with distinct antifungal effector and immunomodulatory functions (52). Although much remains to be explored on the interaction of thymosin α 1 with cells of the innate immune system, the exploitation of TLRs on these cells may represent a new option for therapeutic strategies against fungal infections.

In conclusion, the unique nature of the *Aspergillus* infection, a saprophytic fungus colonizing immunocompromised hosts, demands for a better understanding of immunological mechanisms required to efficiently oppose fungal infectivity that may help optimizing the efficacy of antifungal therapy, as suggested (53, 54). This study shows that the deliberate targeting of cells and pathways of cell-mediated immunity to *Aspergillus* may increase resistance to the fungus and qualifies thymosin α 1 as a promising candidate adjuvant promoting Th1 reactivity to the fungus.

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Figure 1. Thymosin α 1 activates murine dendritic cells upon exposure to *A. fumigatus* conidia.

Purified dendritic cells from lungs of BALB/c were exposed in vitro to *Aspergillus* conidia in the presence of 100 μ g/ml thymosin for 24 h before harvesting cells for phenotypic analysis (A) or assessment of the frequencies of cytokine-producing cells by ELISPOT assay (B). Cell surface expression of MHC Class II antigens, CD80, CD86 and CD40 costimulatory molecules was assessed by FACS analysis. None, unexposed cells. The numbers refer to the median fluorescence intensity. Shown are the data from one representative experiment out of three. For the frequency of cytokine-producing cells, values are the mean \pm SE of samples from 3-5 experiments. * P < 0.05, thymosin-exposed vs unexposed cells.

Figure 2. Thymosin α 1 activates TLRs, p38 MAPK and NF- κ B in murine dendritic cells.

Lung dendritic cells were incubated with unopsonized *Aspergillus* conidia and/or 100 μ g/ml thymosin α 1 for 20-30 min at 37°C before the assessment of TLR expression by RT-PCR and activation of NF- κ B and p38 on cell lysate by probing with specific anti-phospho-38 and anti-NF- κ B Ab, as described in Material and Methods. None, cells exposed to medium alone. Shown are the data from one representative experiment out of three.

Figure 3. Thymosin α 1 activates human dendritic cells for phagocytosis of and cytokine production to *A. fumigatus* conidia.

Immature (im) and mature (m) DC1 and DC2 were obtained from CD11c⁺ blood mononuclear cells as described in Material and Methods. DCs were exposed to 100 μ g/ml thymosin α 1 for 4 h before the exposure to unopsonized conidia for 60 min or 24 h for the assessment of phagocytosis and cytokine production, respectively. Internalization was visualized by light microscopy, the data are the means of several independent experiments and expressed as %

internalization (y axes). The SEs, always below 8, were omitted. Cytokine levels were determined in the culture supernatants by cytokine-specific ELISA. $*P < 0.05$, cytokine production in thymosin-treated vs untreated cells. Cytokine levels in unexposed cells or cells exposed to thymosin alone were below the detection limits of the assays.

Figure 4. Thymosin α 1 protects mice from invasive aspergillosis.

Bone marrow-transplanted (A) or cyclophosphamide (150 mg/kg intraperitoneally, 1 day before the infection)-treated BALB/c (B) mice were infected intranasally with *Aspergillus* conidia and treated with 400 μ g/kg thymosin α 1 intraperitoneally, as described in the Material and Methods. Irradiated recipient C57BL6 mice infused with T-depleted bone marrow cells from BALB/c mice were infected with *Aspergillus* conidia a week after the bone marrow infusion. The fungal growth was assessed in the lungs 3 days after the last conidia inoculation.

Figure 5. Thymosin α 1 decreases inflammatory pathology in mice with invasive aspergillosis.

Periodic acid-Schiff-stained sections were prepared from lungs of BALB/c mice infected intranasal with *Aspergillus* conidia and either left untreated (A) or treated with 400 μ g/kg thymosin α 1 (B) for 6 consecutive days, beginning the day before the infection. Lungs were taken 3 days after the last conidia inoculation of mice immunosuppressed with cyclophosphamide 1 day before the infection. Signs of bronchial wall damage and peribronchial necrosis associated with inflammatory polymorphonuclear cells and fungi were present in untreated mice (A), as opposed to the presence of few lesions with infiltration of mononuclear cells in the peribronchial region of cured mice (B).

Figure 6. Thymosin α 1 accelerates functional recovery of immune cells in mice with invasive aspergillosis.

Flow cytometric analysis of lung cells from bone marrow-transplanted (A) or cyclophosphamide (B)-treated mice, 3 days after the last intranasal inoculation of *Aspergillus* conidia. Treatment with thymosin α 1 was done as described in Material and Methods. The numbers in the upper right corner refer to % of positive cells.

Figure 7. Thymosin α 1 induces antigen-specific lymphoproliferation in mice with invasive aspergillosis.

Lung CD4⁺ T lymphocytes, purified from cyclophosphamide-treated mice 3 days after the last intranasal inoculation of *Aspergillus* conidia, were stimulated with Concanavalin A (C) or heat inactivated *Aspergillus* (D) and dendritic cells, as detailed in the Material and Methods, for 5 days at 37°C before staining with CFSE and FACS analysis. Thymosin α 1 (400 μ g/kg) was given intraperitoneally for 6 consecutive days, beginning the day before the infection. Shown are the results from one representative experiment out of three. A, CD4⁺ T cells alone; B, CD4⁺ T cells and dendritic cells only. The numbers refer to the median fluorescence intensity.

Figure 8. Thymosin α 1 induces the activation of CD4⁺ Th1 cells in mice with invasive aspergillosis.

CD4⁺ T lymphocytes were purified from lungs of bone marrow-transplanted (A) or cyclophosphamide (B)-treated mice, 3 days after the last intranasal inoculation of *Aspergillus* conidia. Infection and treatment were done as in legend to Figure 3. Cells were enumerated by ELISPOT assays. * $P < 0.05$, thymosin-treated vs untreated mice.

Figure 9. Thymosin α 1 increases the phagocytosis and antifungal activity of effector phagocytes.

Bronchoalveolar macrophages and peritoneal neutrophils from uninfected mice were pre-exposed to 100 μ g/ml thymosin for 18 h before incubation with *Aspergillus* conidia. The phagocytic and conidiocidal activity of effector cells were assessed as described in Materials and Methods. Values are the mean \pm SE of samples taken from 3-5 experiments. Each sample was assayed in triplicate. *P < 0.05, thymosin-treated vs untreated cells.

Table 1. Thymosin α 1 increases the therapeutic efficacy of Amphotericin B in mice with invasive aspergillosis.

Treatment [*]			MST [†]	Chitin content [§]
Thymosin α 1	Amphotericin B	$\mu\text{g/Kg}$		
-	-	-	4	39.2 \pm 3.5
+	-	100	4	30.5 \pm 4.1
+	-	200	>60	19.3 \pm 3.3
+	-	400	>60	7.6 \pm 1.4
-	+	1000	>60	24.5 \pm 3.6
-	+	4000	>60	15.2 \pm 3.2
+	+	200+1000	>60	12.4 \pm 2.6

^{*} BALB/c mice, immunosuppressed with cyclophosphamide (150 mg/kg) a day before, were injected intranasally with 2×10^7 conidia of *Aspergillus fumigatus* for 3 consecutive days and treated intraperitoneally for 5 consecutive days with thymosin α 1 and/or amphotericin B.

[†] Median survival time (days).

[§] μg of glucosamine/organ, as determined in the lungs three days after the last intranasal injection of conidia.

^{||} $P < 0.05$, (thymosin α 1 and/or amphotericin B-treated vs untreated mice).

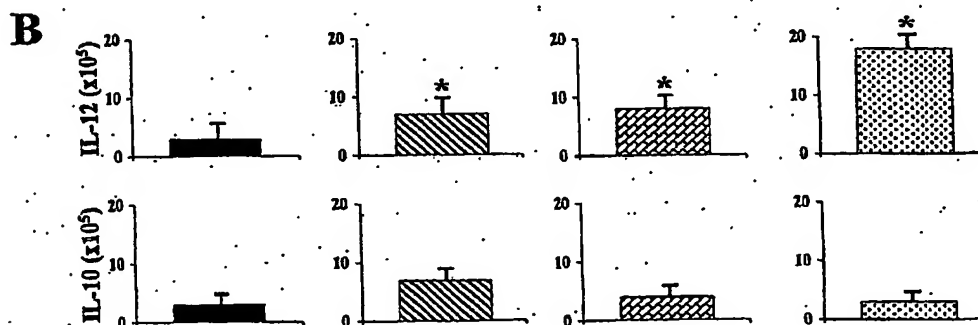
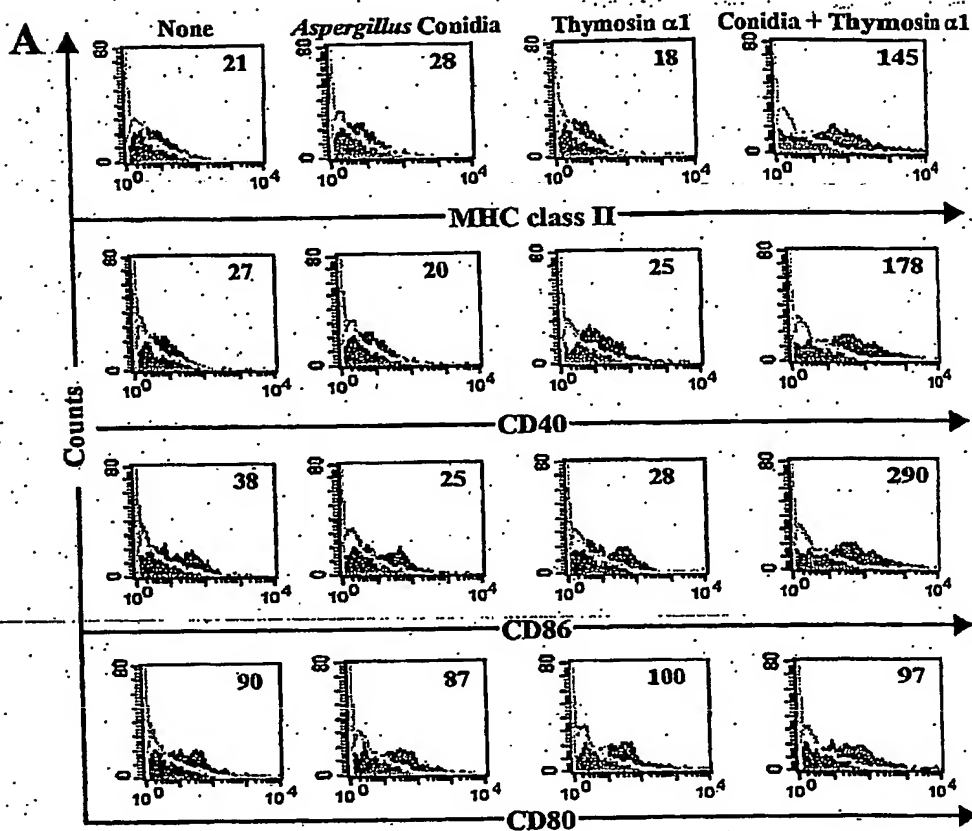


Fig. 1

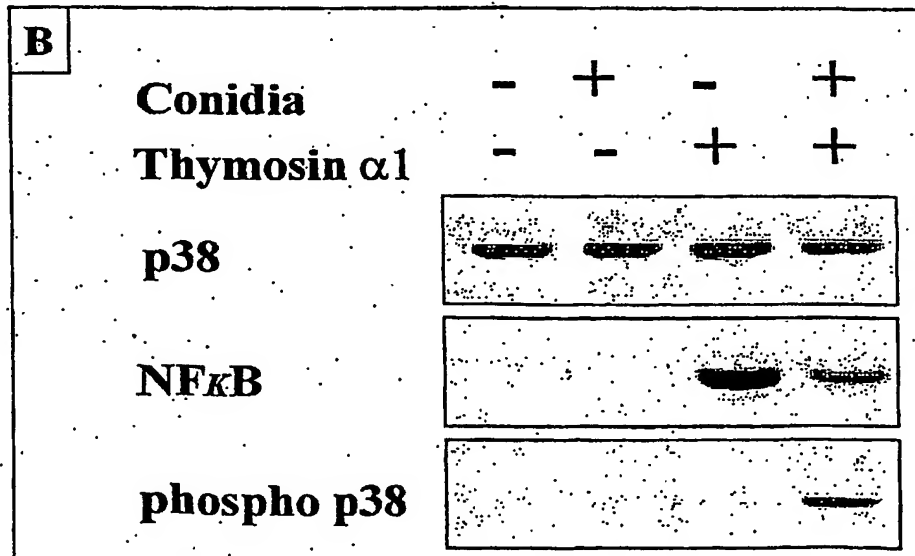
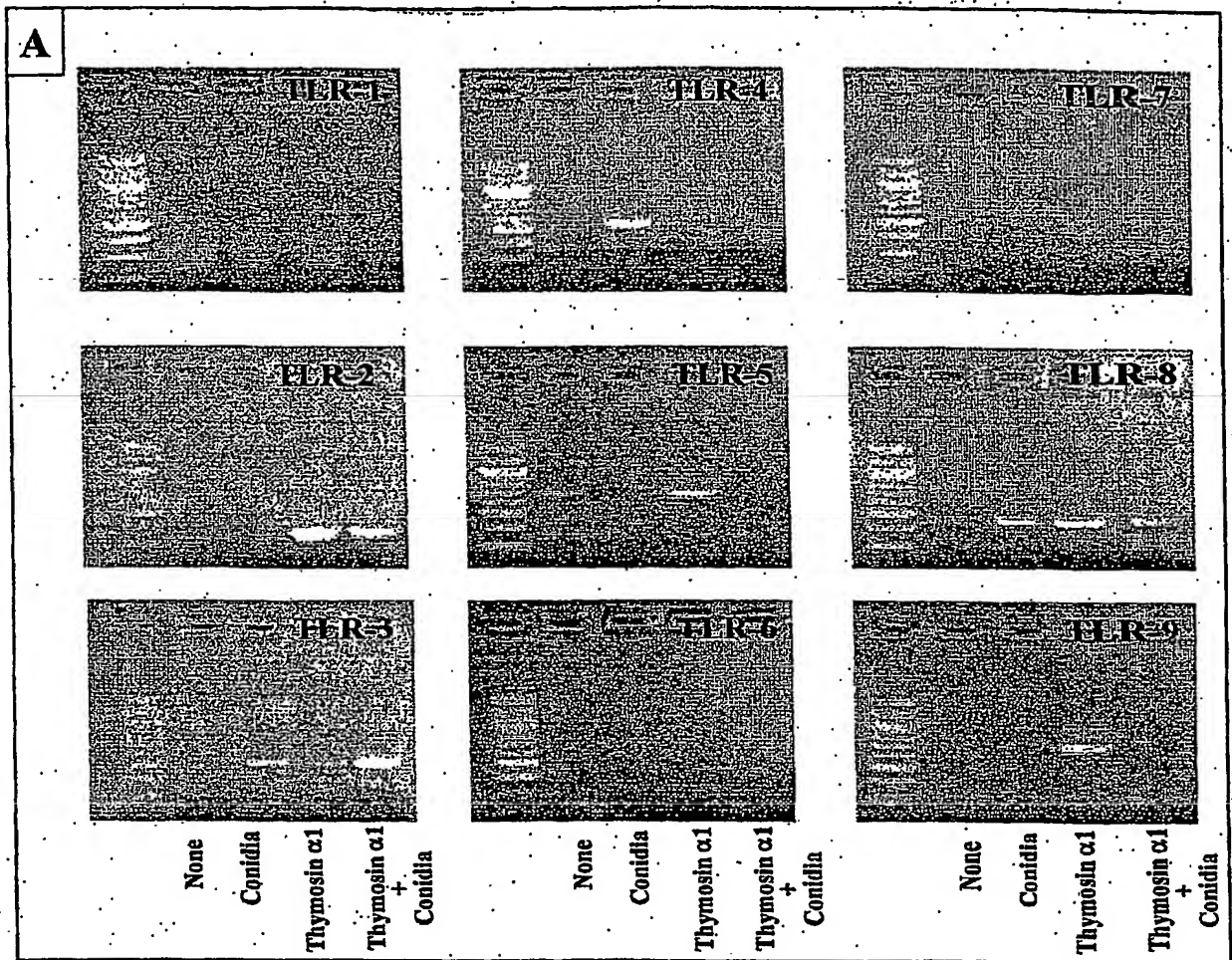


Fig. 2

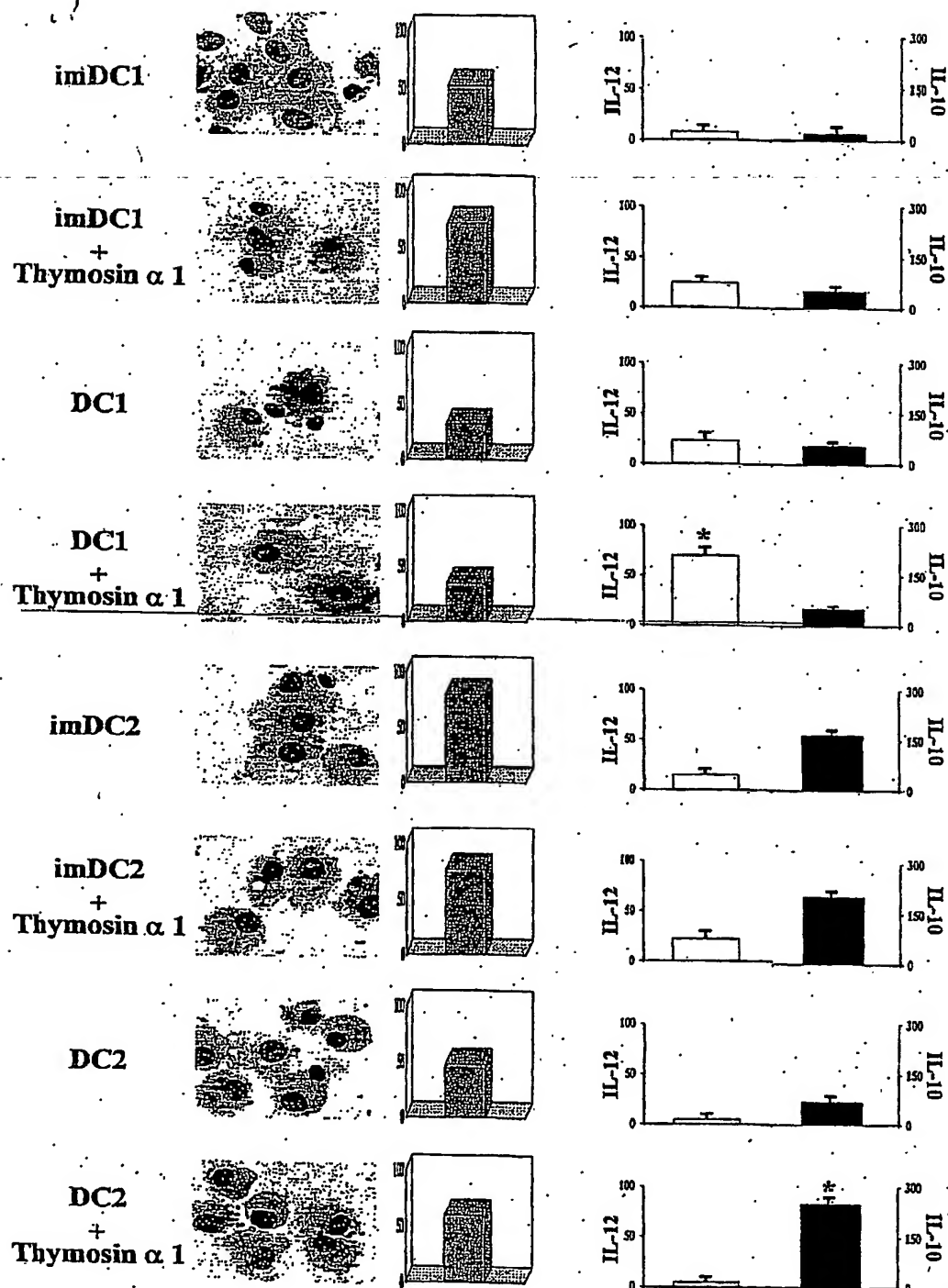


Fig. 3

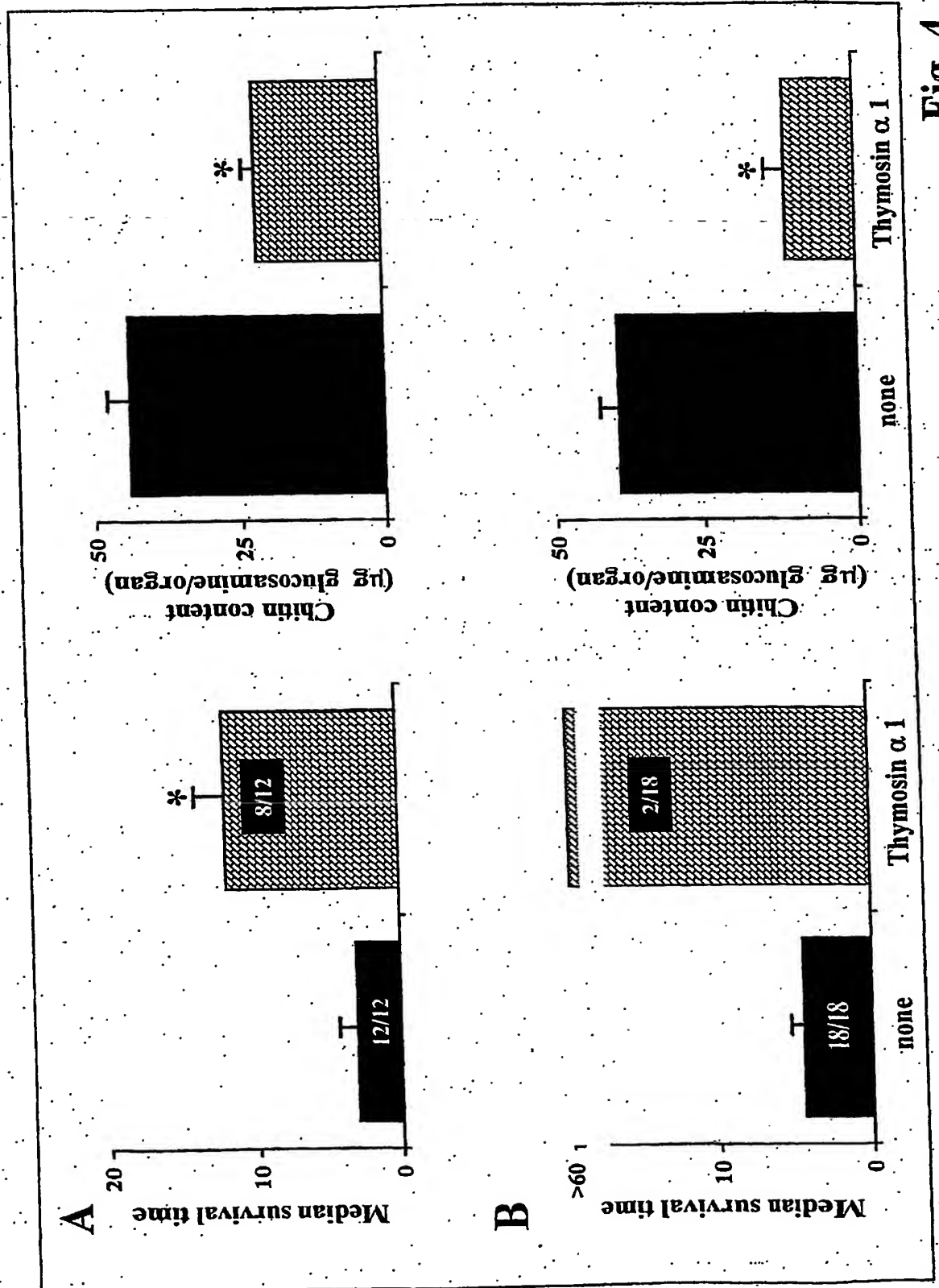
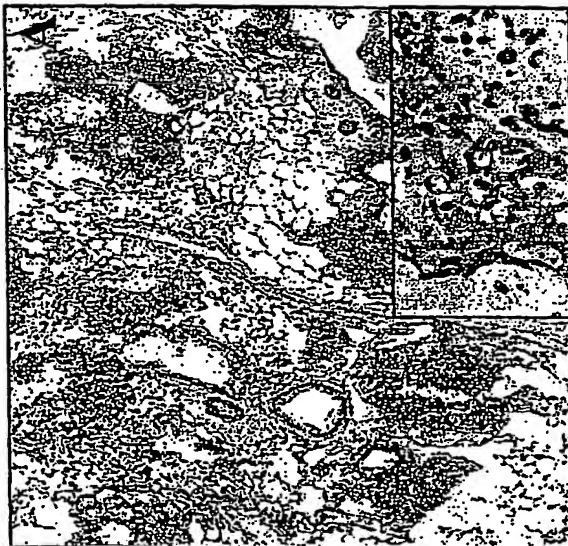
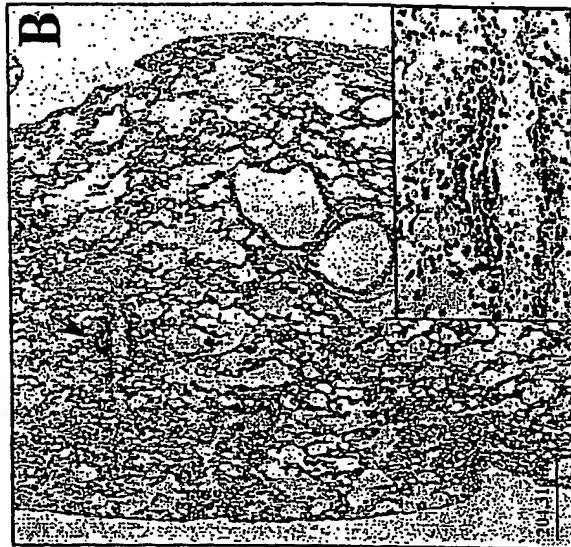


Fig. 4

Fig. 5



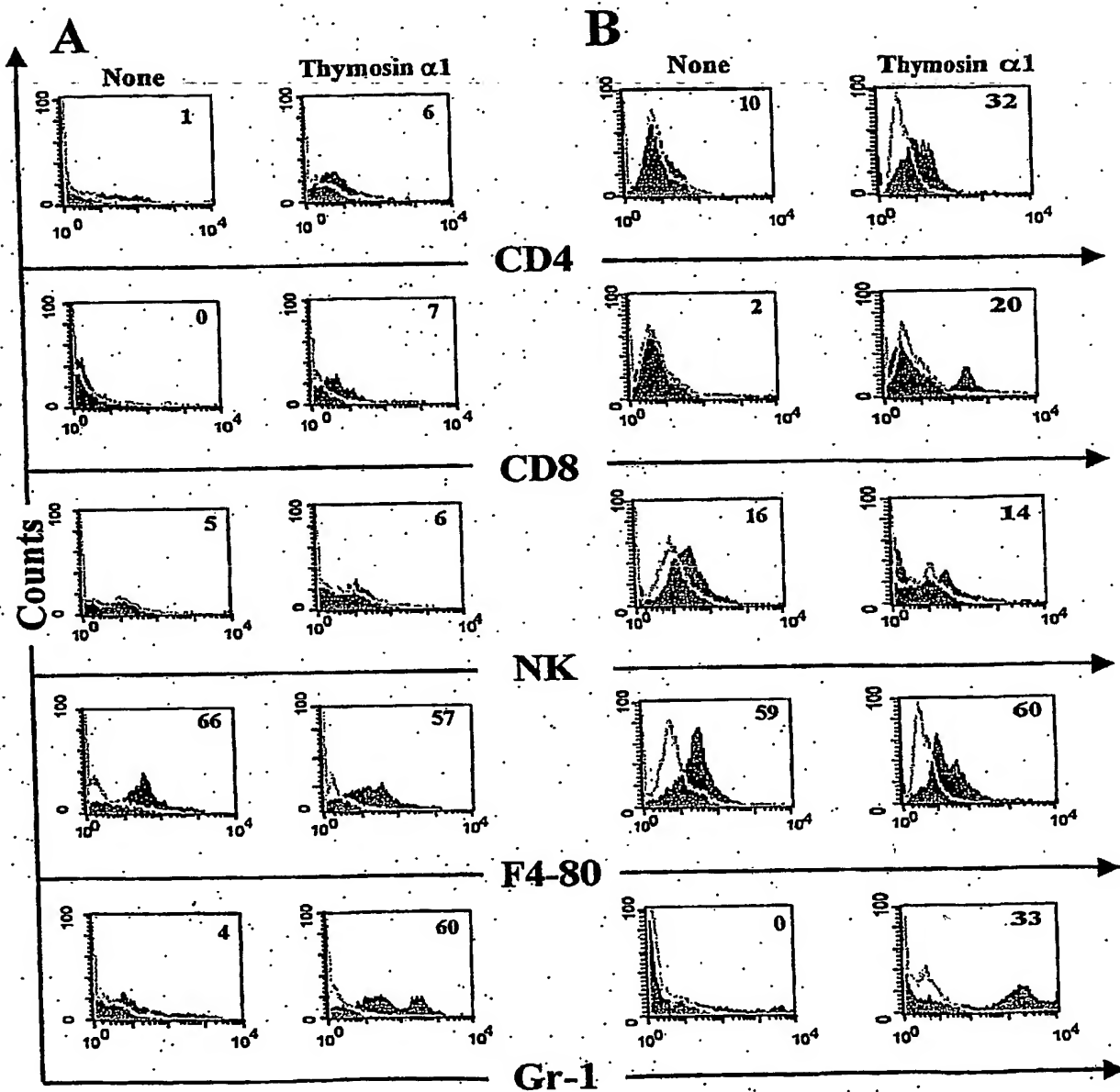
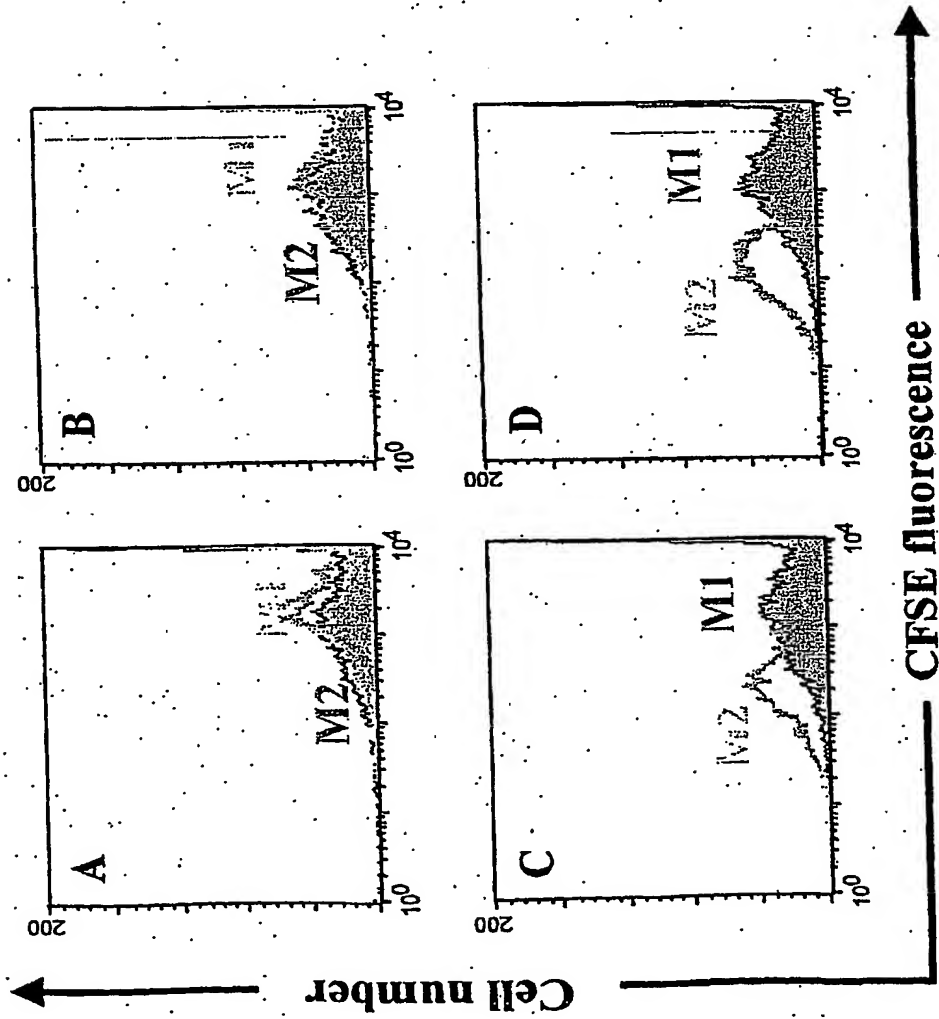


Fig. 6



M1	M2
A 3086	777
B 2565	778
C 3037	772
D 2672	769

Fig. 7

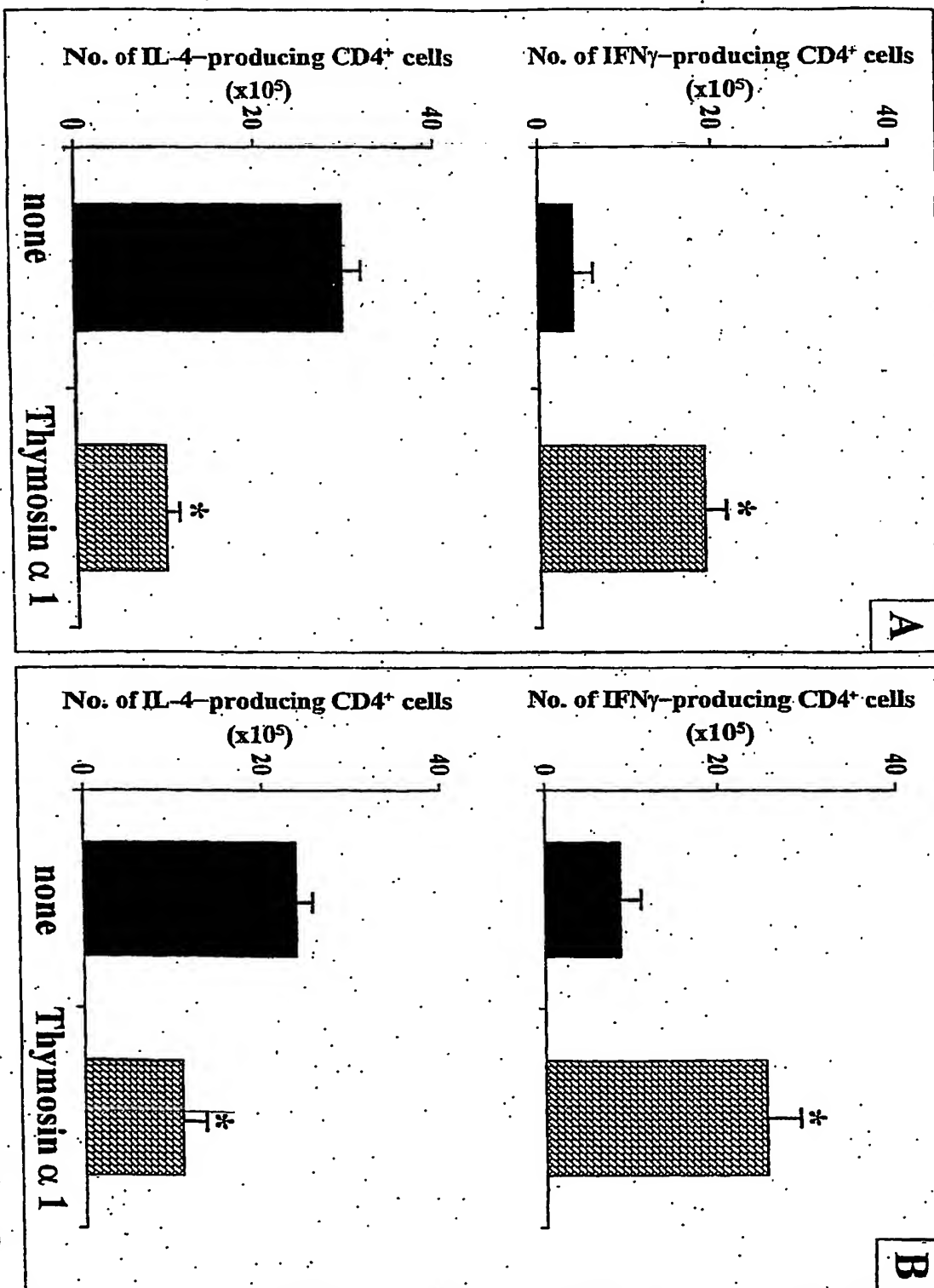


Fig. 8

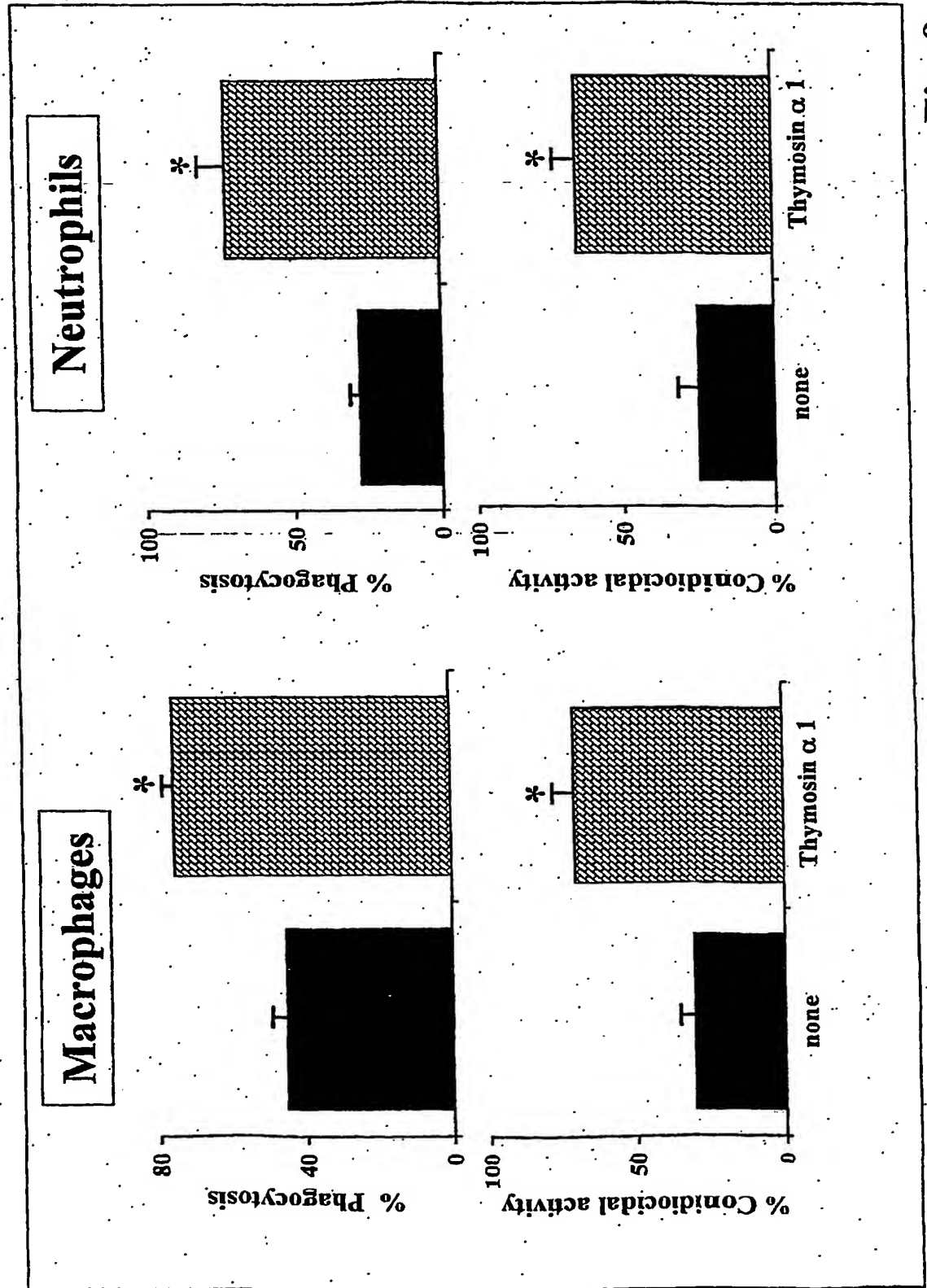


Fig. 9

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